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·Review·

Dysfunction of two lysosome degradation pathways of α-synuclein in Parkinson's disease: potential therapeutic targets?

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Abstract: Parkinson's disease (PD) is pathologically characterized by the presence of α-synuclein (α-syn)-positive intracytoplasmic inclusions named Lewy bodies in the dopaminergic neurons of the substantia nigra. A series of morbid consequences are caused by pathologically high amounts or mutant forms of α-syn, such as defects of membrane trafficking and lipid metabolism. In this review, we consider evidence that both point mutation and overexpression of α-syn result in aberrant degradation in neurons and microglia, and this is associated with the autophagy–lysosome pathway and endosome–lysosome system, leading directly to pathological intracellular aggregation, abnormal externalization and re-internalization cycling (and, in turn, internalization and re-externalization), and exocytosis. Based on these pathological changes, an increasing number of researchers have focused on these new therapeutic targets, aiming at alleviating the pathological accumulation of α-syn and re-establishing normal degradation.

Keywords: Parkinson's disease; α-synuclein; neurodegenerative disease

1 Introduction

Alpha-synuclein (α-syn) is a small cytoplasmic protein that has specific relevance to Parkinson's disease (PD). The α-syn gene comprises six exons, and at least three isoforms are produced by alternative splicing, with different expression patterns and levels[1,2]. Under normal conditions, α-syn exists in a random coil form in the cytoplasm. Although predominantly a neuronal protein, it is also found in glial cells[3]. A portion of this protein has been identified in vesicles and can be secreted from cells into blood plasma and cerebrospinal fluid *via* exocytosis[4,5]. Growing evidence shows that α-syn is involved in the functioning of the neuronal Golgi apparatus and vesicle trafficking, as well as regulating the distribution and fusion of synaptic vesicles[6]. Larsen *et al*.[7] reported increased synaptic vesicle pools in PC12 cells overexpressing α-syn, while α-syn knockout mice have reduced docked and reserve synaptic vesicle pools in hippocampal neurons[8]. All these findings reflect the important functions of α-syn in regulating vesicle traffic.

α-Syn exists as a mix of unstructured, α-helix, and β-sheet-rich conformations in equilibrium, and on the basis of spatial conformation, a study in 2008 suggested that mutations known to enhance aggregation strongly increase the population of the β form. Hence, this could be a conformation associated with pathogenic aggregation[9]. Three missense mutations, A53T, A30P and E46K, as well as multiple copies of wild-type (Wt) α-syn, are linked to hereditary, early-onset PD. Although the molecules and fac-

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tors underlying the intracellular degradation of aggregated α-syn in neurons and microglia remain largely unexplored, accumulating evidence suggests that the conversion of α-syn from soluble monomers to aggregated oligomers and even amyloid-like insoluble forms is the pivotal point in PD pathogenesis due to the abnormal expression of this protein. This would perturb the efficient and correct degradation of α-syn through the usual degradation pathway, which would further exacerbate its toxic effects on neurons. Meanwhile, microglia would be activated and then start a series of inflammatory injury mechanisms, eventually leading to neuronal death. A large body of convincing evidence suggests that abnormal aggregation of α-syn is an important component of PD pathogenesis.

As α-syn is a classical cytosolic protein, its pathogenic effects were assumed to be limited to the cytoplasm of single cells. However, recent studies of extracellular α-syn showed that this protein also has extracellular pathogenic actions. Significantly, a link between the extracellular aggregation of α-syn and the activation of microglia leading to dopaminergic neurotoxicity has been demonstrated in several recent studies *in vivo*[10,11]. Intracellular and extracellular α-syn are necessarily connected; the disruption of intracellular α-syn degradation causes its extracellular secretion. Extracellular α-syn is again internalized by recipient cells[12]. This dynamic relationship will be further described in detail in our review.

2 Externalization and internalization of aggregated α-syn in microglia and neurons

Mutant, overexpressed or oxidatively damaged α-syn can form soluble oligomers or insoluble fibers such as Lewy bodies, which cannot be easily hydrolyzed by the ubiquitin proteasome system. In a study by Su *et al.*, MN9D cells overexpressing Wt α-syn and/or GFP under the regulation of tetracycline were untreated or exposed to doxycycline (DOX), a tetracycline analog that induces α-syn and GFP expression, and only the conditioned medium from $MN9D_{SYN}$ DOX-treated cells contained α-syn[13]. Immunofluorescence showed a significant difference between normal neurons and those overexpressing α-syn: overexpression of α-syn resulted in extensive protein accumulation primarily in cytoplasmic granular structures as well as in punctate structures located along the neuronal processes. α-Syn aggregates in cells when present in abnormal and pathological forms. Furthermore, it can be externalized into the medium and impair the viability of adjacent cells[14].

In a study by Lee *et al.*, differentiated SH-SY5Y cells were treated with 0.2 μmol/L α-syn fibrils for up to 48 h, and they found intracellular accumulation of the internalized α-syn fibrils, which reached a peak at 24 h[12].

Further, ELISA quantification showed that the level of α-syn oligomers is elevated in the body fluids (e.g., cerebrospinal fluid and blood) of PD patients[15]. This suggests that α-syn accumulated in certain neurons is likely to be re-secreted into the extracellular space and then enters the fluid circulation. This re-secretion would be a second strike against adjacent neurons, also involving the activation of microglia and their inflammatory response. The link between externalization and re-internalization of α-syn would initiate the subsequent degradation pathway.

The differences in intracellular trafficking and degradation among different forms of α-syn (monomers, oligomers or insoluble fibrils) in microglia and neurons are evident, and there are differences in temperature-sensitivity and time-dependence. Microglial activation is not only a consequence of α-syn expression by microglia itself but can also be induced by α-syn secreted from neurons and other cells in the brain such as astroglia[11,17]. Moreover, unlike the endocytotic internalization of α-syn fibrils and oligomers, monomeric α-syn enters the cell through membrane transport (a non-endocytotic mechanism), and its internalization is much faster than that of the aggregated form[12]. Consistently, after internalization, monomers exist in the cytoplasm rather than in vesicles[12]. Researchers also found that protein-folding stresses and inhibition of protein quality control increase vesicle translocation and aggregation, indicating that damaged and aggregated proteins are preferentially secreted by cells[12]. That is, α-syn, which has been identified in vesicles, could be transported into the extracellular space *via* an endoplasmic reticulum/

Golgi-independent exocytosis pathway. α-Syn can activate microglia uniquely, with subsequently increased secretion of inflammatory factors such as intracellular reactive oxygen species, tumor necrosis factor-α and prostaglandin E2[13,18]. At this time, microglia plays the role of scavenger, clearing away by phagocytosis the pathological α-syn secreted into the extracellular space. *In vivo*, microglial activation *via* direct interaction with α-syn requires the release of α-syn from cells and subsequent phagocytosis or receptor-mediated activation. Other cells like neurons, SH-SY5Y and COS7 cells take up α-syn by receptor-mediated endocytosis. Although many cells have common receptors for the aggregation of α-syn, it is possible that there are cell type-specific receptors for α-syn aggregates and each type has distinct affinity and uptake kinetics. All of these experimental results indicate that blocked degradation results in pathological excretion, re-internalization and aggregation.

3 Impaired intracellular degradation of α-syn

α-Syn exists in three common forms: monomers, dimers and protofibrils[19]. It is known that α-syn has a tendency to self-assemble under certain circumstances and form dimers and higher-order oligomeric species. Protein misfolding leads to the formation of amyloid-like fibrils, which is the key to Lewy body generation.

For some reasons (e.g., point mutation or injury), α-syn accumulates abnormally rather than entering the normal degradation pathway, thus becoming a potential risk factor. The cytotoxicity of α-syn mostly lies in its soluble or insoluble aggregated oligomers and polymers. Missense mutation, overexpression, or gene damage of α-syn undoubtedly increases the probability of its aggregation. All of these conditions appear to contribute at least partially to familial and sporadic PD[17,18].

Abnormal intracellular α-syn aggregation increases cell toxicity mainly in the following ways: (1) by activating microglia to release inflammatory cytokines; (2) by stalling vesicular fusion (in yeast this specifically causes massive accumulation of vesicles and toxicity associated with disruption of endosome–Golgi trafficking[20]); (3) by interfering with exocytosis and synaptic vesicle recycling in mammalian neurons[21]; and (4) by inducing neurotoxicity (it has been reported that α-syn aggregates transmit pathology *via* neuron-to-neuron interactions[14,22]). Therefore, removal of α-syn from the extracellular space and elimination of its aggregated forms have emerged as critical problems with direct implications for neuronal function and survival.

3.1 Defect of the autophagy–lysosome pathway In cell biology, autophagy or autophagocytosis involves the degradation of a cell's components through the lysosomal machinery. In general, autophagy is divided into macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is the major lysosomal pathway for the degradation of intracytoplasmic proteins. One of its features is the non-specific bulk degradation of cellular contents through both selective and non-selective mechanisms, rather than directly transporting vesicles to lysosomes. In contrast, CMA degrades only soluble proteins, albeit in a selective manner[23].

In the following section, we mainly focus on macroautophagy, together with a description of α-syn degradation by CMA.

3.1.1 Autophagosome: initiation of the autophagy process Mammalian target of rapamycin (mTOR) is a serine/threonine protease which negatively modulates autophagy and apoptosis (mTOR/raptor: forming mTOR complex 1, mTORC1; mTOR/rictor: forming mTOR complex 2, mTORC2). It plays a critical role in regulating cell proliferation and survival, primarily through functioning both downstream and upstream of Akt. Autophagy is a lysosomal degradation pathway that is essential for the regulation of cell survival and death to maintain cellular homeostasis. mTOR is one of the key regulators of the autophagy pathway. Major components in the mTOR axis are inhibitors of the autophagy. Yeast autophagy-related protein 1 (Atg1), the mammalian homologs of which are Unc-51-like kinases 1 (ULK1) and ULK2, can form complexes with many other Atg family members (e.g. Atg13) and then participate in the formation of the early autophagosom (Fig.1). mTOR is activated under growing (nutrient-rich) conditions and phosphorylates ULK1 and mAtg13, and this inhibits

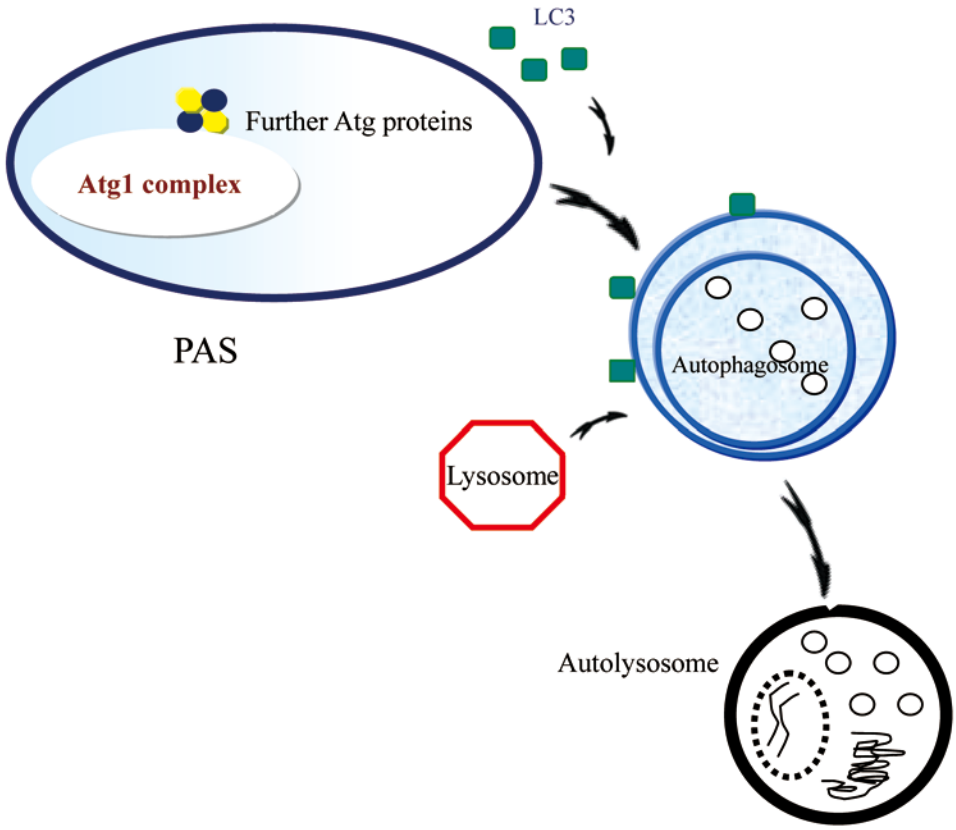

Fig. 1. The formation and maturation of autophagosomes in mammalian cells. Atg1 can associate with some other proteins of the Atg family to form a complex in pre-autophagosomal structure (PAS) with isolation membrane. Then it forms early/initial autophagic vacuole (AVi) with double membrane structure. After the multivesicular endosome and endosomal vesicles fuse with AVi, it turns to be the late/degradative autophagic vacuole (AVd). Autolysosome shapes after lysosome fused with the AVd. Modified from Moreau *et al.* (2011)[24].

ULK1 kinase activity. The inactivation of ULK1 directly causes the failure of assembly of the pre-autophagosomal structure, thus impeding the onset of autophagy and the formation of LC3-II[25,26]. Accordingly, inhibition of mTOR signaling (e.g. with rapamycin) induces autophagy[27]. mTOR mainly functions to act as the inhibitory signal that shuts off autophagy in the presence of growth factors and abundant nutrients[28]. Thus, accumulation of abberant α-syn, which can not be successfully degraded by autophagy, further interferes with fusion of lysosomes and formation of autophagosomes[29,30]. This is in accord with the theory of Crews and and co-workers. On the other hand, activating autophagy with rapamycin or viral-mediated delivery of Atg7 reduces the accumulation of α-syn and ameliorates the associated neuritic alterations[31]. Similar conclusions were also drawn by Spencer's test[16].

LC3-II is essential for the formation of autophagosomes (Fig.1) and measures of its levels have been widely used to estimate their abundance. Data have shown that the macroautophagy marker LC3-II does not co-localize with the large α-syn aggregates either in brain tissue or in cultured cells, suggesting that these aggregates could not be eliminated successfully[32]. LC3-II is decreased in cells overexpressing Wt α-syn, while the PD-associated α-syn mutants A53T and A30P show no such effect[19]. While mTOR participates in the early stage of macroautophagy, double-immunolabeling analysis showed that mTOR and LC3-II increase in cells with the accumulation of α-syn, and both co-localize with aggregated α-syn[31]. Mutant forms of α-syn found in familial PD patients have been

shown to block autophagy. The impairing effect of A53T and A30P mutant α-syn on autophagy may be ameliorated through degradation, albeit in a much lower efficient, of the mutants by CMA targeting[33].

Upon completion of the double-membrane structure of the autophagosome, Atg4 removes LC3-II from the outer autophagosomal membrane in a process called de-conjugation. But LC3-II remains associated with the inner membrane and thus becomes partially degraded after fusion with the lysosome[26]. Bafilomycin A1 blocks the degradation of LC3-II, and this allows the specific assessment of LC3-II formation rates. However, LC3-II levels still decrease in cells overexpressing α-syn in the presence of bafilomycin A1. Therefore, Winslow concluded that the decrease in LC3-II levels caused by α-syn is a result of impaired autophagosome synthesis rather than changes in the function of autolysosomes or lysosomes[19]. *In vivo,* the effect of the α-syn transgene on LC3-II levels is dose-dependent in the mouse brain[19]. However, the author could not attribute this to a reduction of autophagosome formation or an increase of delivery from antophagosome to lysosome[19]. Using an immunofluorescence method, Spencer *et al.* found that compared to the lentivirus (LV) control, abundant, enlarged LC3-GFP structures >1 μm in diameter occurred in serum-starved LV-α-syn neurons[16]. So we can conclude that overexpressing α-syn destroys or changes the normal pathway of autophagy, and these assays reveal a decrease in omegasome formation (the omegasome is a newly characterized structure thought to be the precursor of the forming autophagosome)[19].

3.1.2 The lysosome: late in the autophagy process Lysosomal dysfunction causes a dramatic increase in total α-syn release into the cellular medium, as revealed by Western blot and immunofluorescence[34]. Immunofluorescent staining of α-syn in SH-SY5Y cells overexpressing Wt α-syn show peri-nuclear accumulation of α-syn[34]. As noted above, secreted α-syn causes neuropathological changes and neuronal death.

Despite the demonstration of CMA blockade by mutant α-syns *in vitro*, it is still unclear to what extent CMA blockade and the resultant lysosomal changes are related to the toxicity conferred by aberrant α-syn. Xilouri *et al.* stated that over-expression of A53T α-syn in human SH-SY5Y cells is responsible for the decrease in total lysosomal protein degradation[30]. Also interesting is that over-expression of human A53T α-syn in rat primary neurons causes a significant reduction of CMA activity, followed by a compensatory increase in macroautophagy. Thus, the best guess is that there is some kind of contact between macroautophagy and CMA, which may be in a dynamic, complementary balance. Overexpression of Wt or A53T α-syn acts as an inducing factor that activates the scavenging capacity of these two kinds of autophagy, but this aberrant protein ultimately impairs the normal autophagy–lysosome pathway.

3.2 Endosome–lysosome pathway The endosome is a compartment of the endocytic membrane transport pathway from the plasma membrane to the lysosome. It can be subdivided into the early, late and recycling endosome. How proteins or other lysosome-directed macromolecules such as membrane receptors transit from endosomes to lysosomes is an area of interest. Molecules internalized from the plasma membrane can follow this pathway for degradation, or be recycled to the plasma membrane. Lee *et al*. found that α-syn as oligomers or in amyloid-like insoluble forms can be internalized by cells, and the internalized α-syn co-localizes with EEA1, LAMP1 and caveolin-1 proteins[12]. These proteins are markers of endosomes and lysosomes, or are closely associated with the formation of endosomes. Dynamin is one of the GTPases which is essential for generating endocytic vesicles. Researchers found that the expression of dominant-negative dynamin-1 K44A, which is inactive as a GTPase, blocks endocytosis, resulting in the location of most of the α-syn fibrils on the cell surface, with little cytoplasmic staining[12]. Lee also demonstrated that degradation of α-syn through the lysosome pathway is the main mode. Although much remains to be uncovered in the defective degradation of α-syn *via* the endosome–lysosome pathway, blocking endocytosis or the formation of endocytic vesicles would lead to imperfect degradation of α-syn. Moreover, 25 μmol/L chloroquine which disturbs the membrane trafficking from

endosomes to lysosomes[35], dramatically increases the secretion of α-syn[14]. This is consistent with the theory noted above that the endocytic pathway may be involved in α-syn secretion.

Having confirmed that recycling endosomes are involved in the trafficking of intracellular α-syn, Liu *et al.*[36] proposed another reason for the failure of α-syn elimination by lysosomes[36]. Rab11a, an important member of the Rab GTPase protein family that regulates the function of recycling endosomes, participates in endosome-mediated α-syn exocytosis. In brief, the internalized α-syn can be managed by exocytosis, in addition to the endosome–lysosome-dependent degradation pathway. Further tests using confocal microscopy demonstrated that A53T mutant α-syn co-localizes with Rab11a. α-Syn becomes toxic in yeast upon its association with vesicles forming at the plasma membrane. Thereby, in theory, α-syn enters the vacuolar degradation pathway; however, since it is excluded from the vacuole, α-syn recycles from endosomes back to the plasma membrane[37]. We suppose that there may be some proteins that function to transport and sort specific proteins and guide them into the correct degradation pathway. But this continuous cycle might eventually lead to saturation and blockade of endocytosis and vesicular trafficking routes that merge with the endocytic protein-sorting machinery. Undoubtedly, the endosome–lysosome pathway would be disturbed.

4 Potential new therapeutic targets

One pathological feature of PD—insoluble aggregates composed largely of α-syn in certain populations of neurons and glia—has been attracting increasing concern from researchers. It is clear that when the protein is present in pathologically high amounts, or in mutant forms with enhanced membrane association and oligomerization, it causes neuronal demise with manifestations of impaired neuronal traffic, heightened oxidative stress, mitochondrial degeneration and defects in lipid metabolism. It is essential and beneficial to explore new therapeutic targets from the point of these pathological changes. Here we conclude and summarize some recently proposed hot points for therapeutics.

4.1 Beclin 1 Beclin 1, also known as Atg6, is a coiled-coil, 60-kDa protein. Beclin-1 and its binding partner class III phosphoinositide 3-kinase (PI3K), also named Vps34, are required for the initiation of autophagosome formation in autophagy. Spencer *et al.*[16] proposed that beclin 1 may be a novel therapeutic target for PD treatment, and neuropathological analysis and animal experiments demonstrated that beclin 1 injections ameliorate the synaptic and dendritic pathology in α-syn transgenic mice and reduce the accumulation of α-syn in the limbic system without any significant deleterious effects. This is accompanied by enhanced lysosomal activation and reduced changes in the autophagy pathway (e.g., abnormal aggregation and enlargement of autophagic vesicles marked with LC3-II GFP)[34]. These results imply that a therapeutic approach with rapamycin or beclin 1 might act to re-establish the physiological autophagy–lysosome pathway, thereby playing a positive role in regulating autophagy.

4.2 Gangliosides Gangliosides are composed of a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acids (e.g. n-acetylneuraminic acid) linked to the sugar chain. Natural and semisynthetic gangliosides are considered to be possible therapeutic agents for neurodegenerative disorders[38]. In a study by Wei *et al.*[39], cells overexpressing P123H β-syn were transfected with control vectors, Wt α-syn, or A53T α-syn. Treatment of these cells with *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of glycosyl ceramide synthase, results in various pathological features, including compromised lysosomal activity, enhanced lysosomal membrane permeabilization, and increased cytotoxicity[39]. Consistent with these findings, the expression levels of the lysosomal membrane proteins ATP13A2 and LAMP-2 are significantly decreased. Moreover, the accumulation of both P123H β-syn and α-syn proteins is significant in PDMP-treated cells because of its suppressive effect on autophagy. It is worth re-evaluating the effects of gangliosides on the pathogenesis of PD, especially in terms of axonal pathology.

4.3 Rab GTPase protein family: Rab1 Winslow also found that knockdown of Rab1a inhibits autophagosome

formation and increases the accumulation of autophagy substrates[40]. Besides, Rab1a rescues the inhibitory effect of α-syn on autophagy: overexpression of α-syn markedly reduces vesicle number compared with control conditions, while co-expression of Rab1a-CFP rescues this decrease in vesicle count back to the control level. Therefore, if the gene expression of Rab1 is regulated appropriately, the neurotoxicity caused by aggregated α-syn may be alleviated.

5 Summary

Defective intracellular degradation of α-syn in PD results from a great many of impairments of the degradation progress. Cell-mediated removal of extracellular α-syn may play a crucial role in maintaining an aggregate-free microenvironment and could be a therapeutic target to delay or arrest the progression of neuronal damage. In this review, we discussed the alterations after overexpressing α-syn or in A53T or A30P mutants. α-Syn specifically activates microglia, causing damage to neurons. This protein may also be involved in the internalization–exocytosis–re-internalization cycle, and through this may enter receptive cells intermittently. This process is related to the endosome–lysosome pathway, yet some researchers hold the opinion that intracellularly accumulated proteins are degraded through the autophagic–lysosomal pathway while extracellular proteins pass through the endosomal–lysosomal pathway. Any defect of these two pathways will bring about abnormalities in α-syn degradation, further increasing the cytotoxicity.

In addition, as far as autophagy is concerned, different treatments, such as rapamycin, beclin1, and natural and semisynthetic gangliosides can alleviate the accumulation of α-syn, thus ameliorating the related neuropathology[34]. Overexpressing Rab1a can rescue the decrease in autophagosomes resulting from overexpression of α-syn, while for exocytosis and endocytosis, hindering the interaction between Rab11 and HSP90, or using inhibitors of these two proteins can undoubtedly reduce the extracellular level of α-syn to a certain extent, according to the experiments of Liu *et al.*[36]. It is also possible that as yet unknown drugs or improvement of certain proteins that function to transport and sort specific proteins into the correct degradation pathway, could further reduce the pathological damage in PD.

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